Gram-Scale Preparative HPLC of Phospholipids from Soybean Lecithins[1]

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A preparative procedure has been developed to isolate gram quantities of phospholipid classes from soybean lecithins. Various steps taken to accomplish the isolation are described—an analytical method with silica column and light scattering detector, alcohol fractionation of deoiled lecithins, and columns with increasing internal diameters but packed with the same stationary phase. The loading study showed that it was possible to inject 20 mg on a 100 × 8 mm Radial μPorasil column. The separation was scaled up to a 25-mm i.d. column and finally to a 50-mm i.d. column. With the larger column, 2.1 g of phospholipids were separated. The collected fractions (phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid and phosphatidylcholine) were of high purity (>99%). The solvent consumption was 7.2 L (separation and column equilibration), and a minimum of 10 g of polar lipids can be separated daily.

KEY WORDS: **Analytical HPLC, lecithin, phospholipids, preparative HPLC.**

Phospholipids are functioning phosphatides of cell membranes and are important substances in biomedical, pharmaceutical and nutrition research areas. They are widely used in the food and cosmetics industries, as well as in industrial manufacturing. The main commercial source of phospholipids is plant seeds. The food manufacturers use the term "commercial lecithin" to denote emulsifiers and wetting agents prepared during vegetable oil refining. Yearly, 135,000 tons of lecithins are available worldwide mainly from soybean oil. Commercially available lecithin products represent a complex mixture of neutral lipids, phospholipids, glycolipids and smaller amounts of other substances. During the last decade, a considerable number of analytical high-performance liquid chromatography (HPLC) procedures for separating phospholipid classes have been described (refs. 1–3). Adsorption chromatography in combination with UV detection is the most common method. Christie (4) has published an excellent study on the separation and quantitation of lipid classes by adsorption HPLC and light scattering detection immediately prior to our own results with the same type of detector, but with a diol column (5).

Our purpose was to prepare, for functional properties and biological research, gram quantities of pure phospholipids from soybean lecithins, mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and phosphatidylinositol (PI). To our knowledge, only a few studies have been done on preparative HPLC of phospholipids (6–8). The mobile phases used in preparative separations are complex mixtures of various solvents, such as acetonitrile/methanol/phosphoric acid (6); hexane/isopropanol/water (7); and acetonitrile/isopropanol/methanol/water/trifluoroacetic acid (8). In these experiments, the loading studies carried out on silica columns with diameters as large as 57 mm indicated a column load of 180 mg only. In order to avoid a complex recovery method for isolated phospholipids fractions, we decided to use a mobile phase without acidic or basic components. In this paper, we present a preparative procedure to isolate gram quantities of phospholipids classes from soybean lecithins. The various steps taken to accomplish the preparative HPLC isolation are described.

MATERIALS AND METHODS

Materials. Commercial soybean lecithin (Asol 100) was supplied by Lucas Meyer (Hamburg, Germany). Deoiled lecithin with acetone treatment, alcohol-soluble and alcohol-insoluble lecithin were prepared in our laboratory. Phospholipid standards were purchased from Sigma (St. Louis, MO).

HPLC-grade hexane, water and tetrahydrofuran were purchased from Rathburn (Walkerburn, Scotland). Isopropanol and ethanol 95° RPE-ACS grade were from Carlo Erba (Milan, Italy). Chloroform purex and acetone puran were obtained from SDS (Peypin, France).

Preparation of deoiled soybean lecithin. Deoiled lecithin was prepared from soybean lecithin (70 g) with acetone (500 mL) treatment. The solution was allowed to stand at 4°C for 2 hr. The solution was then filtered and the acetone solution discarded. This treatment was repeated until the acetone layer was colorless. The acetone-insoluble fraction, which contains polar lipids, was carefully dried. With the supplied lecithin, the yield in deoiled lecithin was about 54%.

Alcohol fractionation. Ten grams of deoiled lecithin was mixed with 50 mL of ethanol 95° at 40°C for 30 min. The mixture was allowed to stand and then filtered. The ethanol solution, which was concentrated in a rotary evaporator, was the alcohol-soluble lecithin (yield approx. 60%). The residue from filtration was carefully dried; it constituted the alcohol-insoluble lecithin (yield approx. 40%).

HPLC systems. The preparative HPLC system was a Waters Model Prep LC 3000 (Milford, MA) and consisted of a programmable quaternary solvent proportionating valve with a Rheodyne 7010 injector equipped with a four parts loop (injection volumes from 50 to 5100 μL). Preparative samples (40 mL) were introduced directly through the pump at a flow rate of 10 mL/min. The compounds were detected with a SM 4000 variable UV absorbance detector (LDC Milton Roy, Riviera Beach, FL) equipped with a 35-μL semi-Prep fluid cell. The effluent absorbance was monitored at 210 nm and the sensitivity was set at 1.0 AUFS. The chromatograms were registered on a W+W recorder from Scientific Instruments Inc. (Bale, Switzerland). The columns used to develop the

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TABLE 1

Conditions of Gradient Elution, Flow Rate and Load in Relation to the Geometric Characteristics of the Columns

Column	Gradient[a]					
L × i.d. (mm)	Time (min)	Hexane	iPOH	Water	Flow rate (mL/min)	Load (mg)
100 × 8	0	42	53	5	2	25
	15	39	51	10		
100 × 25	0	42	53	5	20	200 in
	15	39	51	10		10 mL $CHCl_3$
300 × 47	0	42	53	5	80	2100 in
	45	39	51	10		40 mL $CHCl_3$

[a]Concave gradient curve number 7 from Waters between the compositions specified at each time interval.

preparative separation were Silica Radial Pak Cartridges with radial compression cartridge holders (Waters): a) μPorasil cartridge, d_p 10 μm; L, 100 mm; d_c, 8 mm; b) μPorasil cartridge, d_p 10 μm; L, 100 mm; d_c, 25 mm; c) μPorasil cartridge, d_p, 15–20 μm; L, 300 mm; d_c, 47 mm; held in a Waters 1000 PrepPak Module. These columns were protected with homemade guard columns: 20 × 2 mm for the 8-mm i.d. column; 50 × 4.7 mm for the 25-mm i.d. column; each of these was packed with 25–40 μm Techsil silica (HPLC Technology, Cheshire, U.K.). For the preparative column (i.d. 47 mm) a guard column of 180 × 11 mm was packed with silicagel 60–200 μm (Merck, Darmstadt, Germany).

The mobile phase used to perfect and achieve the preparative separations was a mixture of hexane, isopropanol and water. The solvent programs used for the elution of lipid classes from the different columns are summarized in Table 1. The flow rates and amounts of injected samples are also reported.

After the individual phospholipid fractions were collected, the solvents were removed under vacuum at 40°C, and the resulting fractions were then evaluated for purity.

The system used for analytical work and to monitor the preparative HPLC fractions was a LC 5500 from Varian (Sunnyvale, CA) and consisted of a programmable ternary solvent proportioning valve; a Rheodyne valve injector was equipped with a 10-μL loop. A laserlight scattering detector (L/LSD) from Varex (Rockville, MD) operated with an internal nitrogen pressure of 32 psi. The temperature of drift tube was set at 70°C and the sensitivity was fixed at range 5. A 100 × 4.6 mm i.d. column packed with Spherisorb 3 μm silica (Hichrom, Reading, U.K.) and a guard column 2 × 0.2 cm packed with Techsil silica were used. The detector was attached to a Vista 402 chromatographic station (Varian) for recording and integrating the eluted peaks. The mobile phase used was that reported in an earlier study by Christie (4), but with modification of the gradient time running. Solvent A is a mixture of hexane and tetrahydrofuran (99:1, v/v), solvent B is isopropanol/chloroform (80:20, v/v), and solvent C is a mixture of isopropanol/water in the proportions 50:50 (v/v). The ternary gradient elution is shown in Table 2. The flow rate was 1 mL/min throughout. Lipid samples (10 mg) were dissolved in 1 mL chloroform and the solution was sonicated for 30 seconds before injection.

For both analytical and preparative work, when the stored columns were to be reused, they were first equilibrated, prior to analysis, with the more polar mobile phase and then reequilibrated with the initial solvent. In these conditions, no changes were observed in the elution times of individual components.

TABLE 2

Gradient Elution System Required for the Analytical HPLC Method

Time	Solvents (%)		
(min)	A[a]	B[b]	C[c]
0	100	—	—
1	100	—	—
5	80	20	—
5.1	42	52	6
20	32	52	16
30	32	52	16
34	30	70	—
37	100	—	—

[a]Denotes mixture of hexane/tetrahydrofuran, 99:1 (v/v).
[b]Denotes mixture of isopropanol/chloroform, 80:20 (v/v).
[c]Denotes mixture of isopropanol/water, 50:50 (v/v).

RESULTS AND DISCUSSION

The type of analytical separation that was achieved with the solvent gradient described as detected by laser light scattering is illustrated by Figure 1, where the deoiled Asol 100 lecithin was the example. The main phospholipids were well resolved and the lysophosphatidylcholine was eluted within 30 min.

The compositions of deoiled lecithin, the alcohol-soluble lecithin and the alcohol-insoluble fraction were investigated by analytical HPLC procedure. The results are reported in Table 3. The soybean lecithin had a high content in PC; it was a phosphatidylcholine-enriched soybean lecithin. The alcohol-soluble fraction was mainly enriched in PC and LPC, whereas PI was entirely removed. PI and PA were concentrated in the alcohol-insoluble fraction. The compositions of these fractions always showed the same partition, but with variations in percentage of each individual PL, depending on experimental conditions.

The working sample for preparative isolation was chosen for two reasons. First, we wanted to obtain the optimal throughput per injection for each individual phos-

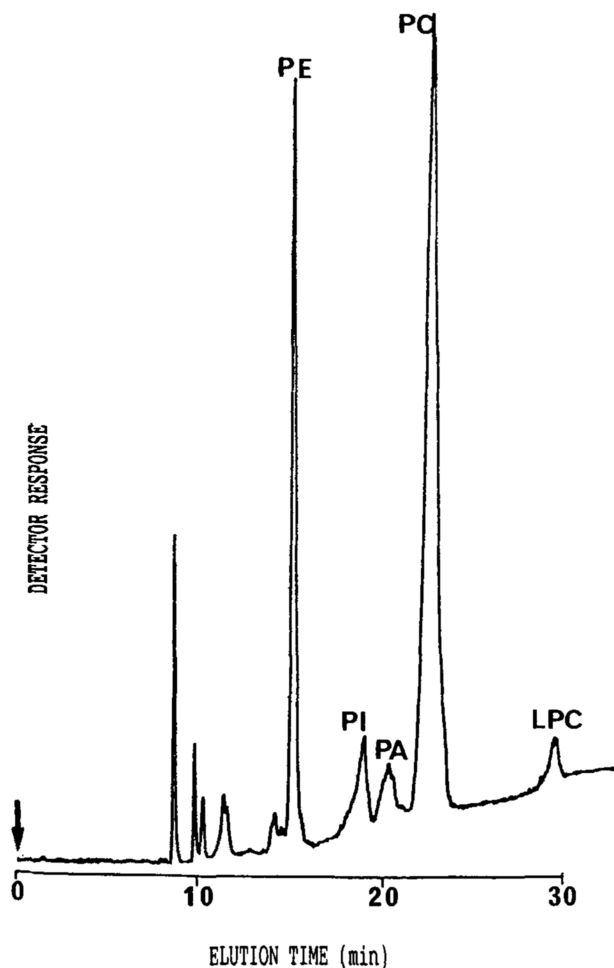

FIG. 1. Analytical separation of deoiled lecithin by HPLC with silica Spherisorb column and laserlight scattering detector. Abbreviations: PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; and LPC, lysophosphatidylcholine.

pholipid; and second, we wanted to develop the separation system on the more complex mixture. For these reasons, the alcohol-insoluble lecithin was retained.

As the columns were packed with the same stationary phase and differed only in internal diameter and in length for the larger column, the first step was to develop the separation in the 100 × 8 mm μPorasil radial column. Different mobile phases have been tested to achieve separation between the phospholipids classes. Single solvents such as ethanol, isopropanol and methanol, preferred for an easier further recovery of separated compounds, did not permit the elution of PC and LPC, which are the more polar phospholipids.

As recommended by several authors (9-11), it was necessary to add increasing amounts of water in alcohol. To achieve the separation between less polar compounds (neutral lipids, glycolipids) and phospholipids, the incorporation of nonpolar solvent in the hydroalcoholic mixture, such as hexane, was indispensable. Finally, the polarity of the solvent system was varied by changing the

TABLE 3

Typical Compositions of the Major Phospholipids in Soybean Lecithin Asol 100 and the Alcohol-Extracted Fractions[a]

Compound[b]	Soybean lecithin	Alcohol-soluble	Alcohol-insoluble
PE	20.0	12.3	30.7
PI	11.0	—	23.8
PA	7.0	5.8	12.4
PC	43.7	65.0	17.0
LPC	1.0	3.0	—
Others[c]	17.3	13.9	16.1

[a]Amounts are expressed as weight percent.

[b]Abbreviations: PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; and LPC, lysophosphatidylcholine.

[c]Neutral lipids and glycolipids mainly.

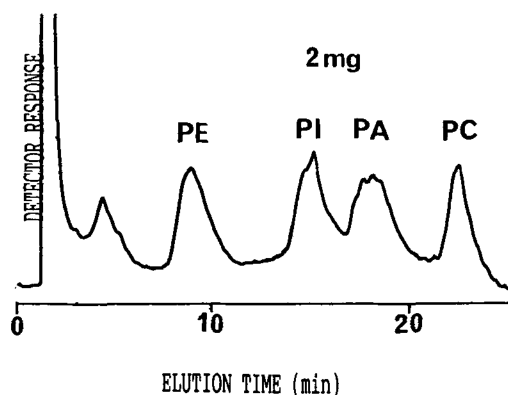

FIG. 2. Separation of 2 mg of the alcohol-insoluble lecithin on the μPorasil cartridge: L, 100 mm; d_c, 8 mm; d_p, 10 μm. Abbreviations as in Figure 1.

amount of water, whereas the hexane/isopropanol ratio was kept almost constant. Figure 2 shows the separation of the alcohol-insoluble lecithin, the amount of injected sample was 2 mg. As can be seen, an efficient separation of PE, PI, PA and PC is achieved within 25 min. The capacity factors range from 4.6 for PE to 13.0 for PC. The α value (selectivity) is smallest for the PI/PA pair (α=1.2). As a pure state was desired for all phospholipids, it meant that the PI/PA pair will determine the maximum allowable compound size.

The loading study was carried out by injecting increasing amounts of alcohol-insoluble lecithin—4, 8, 16, 21 and 30 mg. Due to the limited solubility of phospholipids in chloroform, the column was overloaded in volume; for the injection of 21 mg, the sample was dissolved in 2.6 mL of chloroform. The separation (Fig. 3) between PI and PA is not so efficient because eluted peaks are significantly wider than those from the analytical sample (2 mg). Nevertheless, by collecting fractions it was possible to isolate pure PI and pure PA. For larger sample sizes, the column was overloaded and it became difficult to isolate pure phospholipids with good recovery.

The separation was next scaled up for use with the

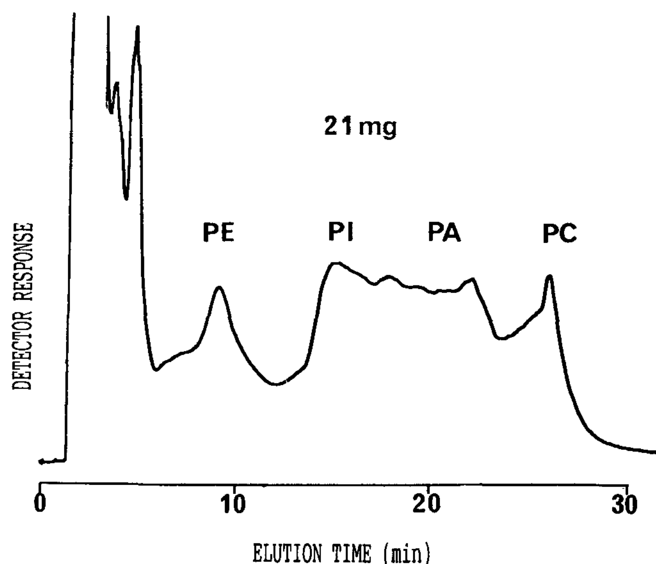

FIG. 3. Separation of 21 mg of the alcohol-insoluble lecithin on the μPorasil cartridge: L, 100 mm; d_c, 8 mm; d_p, 10 μm.

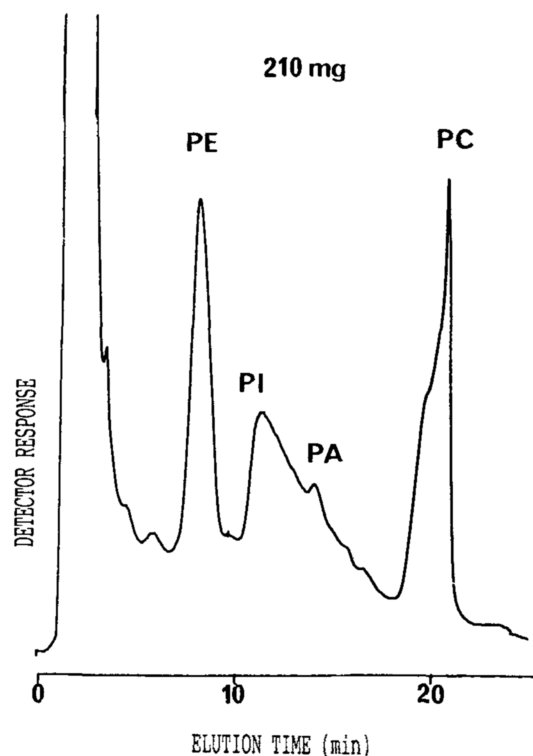

FIG. 4. Separation of 210 mg of the alcohol-insoluble lecithin on the μPorasil cartridge: L, 100 mm; d_c, 25 mm; d_p, 10 μm.

25-mm i.d. column, which was otherwise identical to the analytical column. Because the cross-sectional area of the 25-mm i.d. column was almost ten times larger, the sample size was increased to 210 mg. The flow rate was similarly increased to 20 mL/min. The resulting separation is shown in Figure 4. As can be seen, the separation is almost better than the separation with the 8-mm i.d. column. It was possible to isolate pure phospholipids.

The further scale-up of this procedure had been applied to the 47-mm i.d. column. Because the cross sectional area

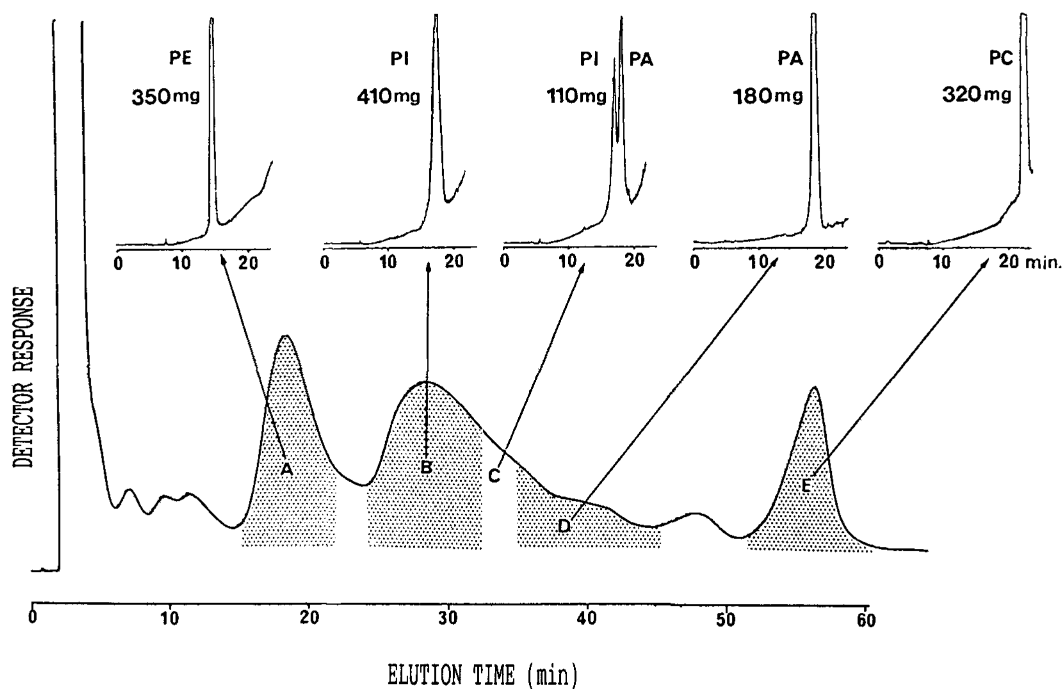

FIG. 5. Separation of 2100 mg of the alcohol-insoluble lecithin on the μPorasil cartridge: L, 300 mm; d_c, 47 mm; d_p, 15–20 μm. Shaded areas indicate the collected fractions; the analytical chromatogram, the purity and the recovery are indicated for each fraction.

of this column is three and half times larger when compared with the 25-mm i.d. column, and the length is triple (30 cm instead of 10 cm), the sample load was increased to 2.1 g. The flow rate was increased to 80 mL/min in order to maintain the same linear velocity. Due to the different length of this column, the gradient duration was scaled up to 45 min so that the gradient occurred over the same number of column volumes.

The separation that was achieved with this procedure is illustrated by Figure 5 where 2.1 g of alcohol-insoluble lecithin dissolved in 40 mL of chloroform were injected. Phosphatidylethanolamine and phosphatidylinositol were clearly resolved, phosphatidic acid formed a shoulder, sometimes better resolved, on the phosphatidylinositol peak. Neutral lipids and glycolipids were eluted just ahead of phosphatidylethanolamine. The separation was complete in 60 min with the elution of phosphatidylcholine, and a period of 30 min of further elution was needed to reequilibrate the column with the initial solvent mixture.

It was possible to achieve the objective of purification by fraction cutting. Five fractions were collected as illustrated in Figure 5 by the shaded areas. Their purities were checked by the analytical procedure. The chromatograms of these fractions are shown in Figure 5 above each shaded area. Typically, fraction A after evaporation yielded 350 mg of phosphatidylethanolamine whose analysis showed <1% impurity. A total of 410 mg of phosphatidylinositol (>99%) was recovered from the fraction B. Fraction C was a mixture of PI (43%) and PA (57%) and can be reinjected to improve the purity. Fraction D yielded 180 mg of pure phosphatidic acid (>99% by the analytical procedure) and 320 mg of pure phosphatidylcholine were recovered from fraction E.

A total of 1300–1400 mg of phospholipids can be recovered from the injection of 2100 mg of polar lipids. The four main phospholipids from alcohol-insoluble lecithin have been obtained at better than 98–99% pure. From the weight-percentage composition of lecithin, the mass of the individual fractions and the sample load, it was calculated that 82, 69 and 90%, respectively, of the injected amounts of PI, PA and PC were recovered, whereas only 54% of the total amount of PE could be obtained in such purity.

The solvent consumption was 7.2 L (60 min for separation and 30 min for equilibrium at 80 mL/min.). A minimum of 10 g of polar lipids can be separated daily.

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